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NisT, the Transporter of the Lantibiotic Nisin, Can Transport Fully Modified, Dehydrated, and Unmodified Prenisin and Fusions of the Leader Peptide with Non-lantibiotic Peptides*

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Lantibiotics are lanthionine-containing peptide antibiotics. Nisin, encoded by *nisA*, is a pentacyclic lantibiotic produced by some *Lactococcus lactis* strains. Its thioether rings are posttranslationally introduced by a membrane-bound enzyme complex. This complex is composed of three enzymes: NisB, which dehydrates serines and threonines; NisC, which couples these dehydrated residues to cysteines, thus forming thioether rings; and the transporter NisT. We followed the activity of various combinations of the nisin enzymes by measuring export of secreted peptides using antibodies against the leader peptide and mass spectroscopy for detection. *L. lactis* expressing the *nisABTC* genes efficiently produced fully posttranslationally modified prenisin. Strikingly, *L. lactis* expressing the *nisBT* genes could produce dehydrated prenisin without thioether rings and a dehydrated form of a non-lantibiotic peptide. In the absence of the biosynthetic NisBC enzymes, the NisT transporter was capable of excreting unmodified prenisin and fusions of the leader peptide with non-lantibiotic peptides. Our data show that NisT specifies a broad spectrum (poly)peptide transporter that can function either in conjunction with or independently from the biosynthetic genes. NisT secretes both unmodified and partially or fully posttranslationally modified forms of prenisin and non-lantibiotic peptides. These results open the way for efficient production of a wide range of peptides with increased stability or novel bioactivities.

A wide spectrum of biological functions, such as hormone, growth factor, enzyme inhibitor, antigen, antibiotic, and ionophore, can be found among peptides. Cyclization of peptides has been shown to be a valuable method to obtain biostable analogs. Furthermore, by conformational constraints, enhanced or modulated receptor interaction can be obtained (1, 2). Thioether rings can contribute to enhanced peptide stability, enhanced resistance against proteolytic degradation (3, 4), and modulation of receptor interaction (2).

Lantibiotics are bacterial peptides with intramolecular thioether bridges (5). They owe their name to their antibiotic activities and the presence of lanthionine residues. Lanthionines are thioether-containing amino acids. A variety of activities

have been demonstrated for lantibiotics, e.g. the autoinduction of lantibiotic synthesis (6), permeabilization of target membranes (7–11), inhibition of cell wall synthesis (12), lipid II binding (13), inhibition of phospholipase A₂ (14), and modulation of autolytic enzymes (15) and of an angiotensin-converting enzyme (16). These activities all depend on the presence of thioether rings. By controlling the lanthionine-synthesizing enzyme complex, one might envisage the possibility of introducing thioether rings at any peptide position. However, at present, only one new thioether ring has been synthesized in a lantibiotic (3). Most interestingly, *in vitro* activity of the lanthionine-synthesizing enzyme LctM has recently been demonstrated (17).

The best known lantibiotic is nisin, which is produced by some *Lactococcus lactis* strains. Nisin is widely applied as a food preservative (18) because of its antimicrobial activity. It displays a variety of antibiotic activities against many Gram-positive bacteria (7, 19). Breukink *et al.* (13) found that nisin interacts with lipid II of the target cell and, already at very low concentrations, this complex permeabilizes the membranes for small ions and solutes.

Nisin is composed of 4 methylanthionines, 1 lanthionine, 2 dehydroalanines, 1 dehydrobutyrine, and 26 unmodified amino acids (20, 21). At position 33, mostly a dehydroalanine is present, but in some cases, an unmodified serine can be found (22). The above-mentioned uncommon residues are posttranslationally produced by intracellular membrane-associated enzyme complexes (23, 24). The enzyme NisB dehydrates serines and threonines; NisC is responsible for thioether bridge formation by coupling the dehydro residues to cysteines, and NisT exports fully modified prenisin. The extracellular serine protease NisP cleaves off the N-terminal 22–23 amino acid leader peptide (with or without initiating methionine), whereupon the active nisin is released (Fig. 1).

Here we show that the nisin-precursor transporter NisT, in different combinations with NisB and NisC, is able to transport a wide variety of modified and unmodified peptides. This now opens the way to the biotechnological production of modified peptides with novel bioactivities and improved stability. Moreover, we show that the processing enzyme, NisP, requires lanthionine formation of the propeptide for proper functioning.

MATERIALS AND METHODS

Leader Peptide—A synthetic leader peptide without initiating methionine was purchased from Synpep, Dublin, CA. Bacterially produced leader peptide was purified by binding to Teflon beads, a C18 column, and elution with an acetonitrile gradient. Cleavage of leader peptide from fully modified prenisin resulted from a 15-min incubation at 37 °C with 1 mg/ml trypsin.

Anti-leader Peptide Antibodies—Polyclonal antibodies were raised in

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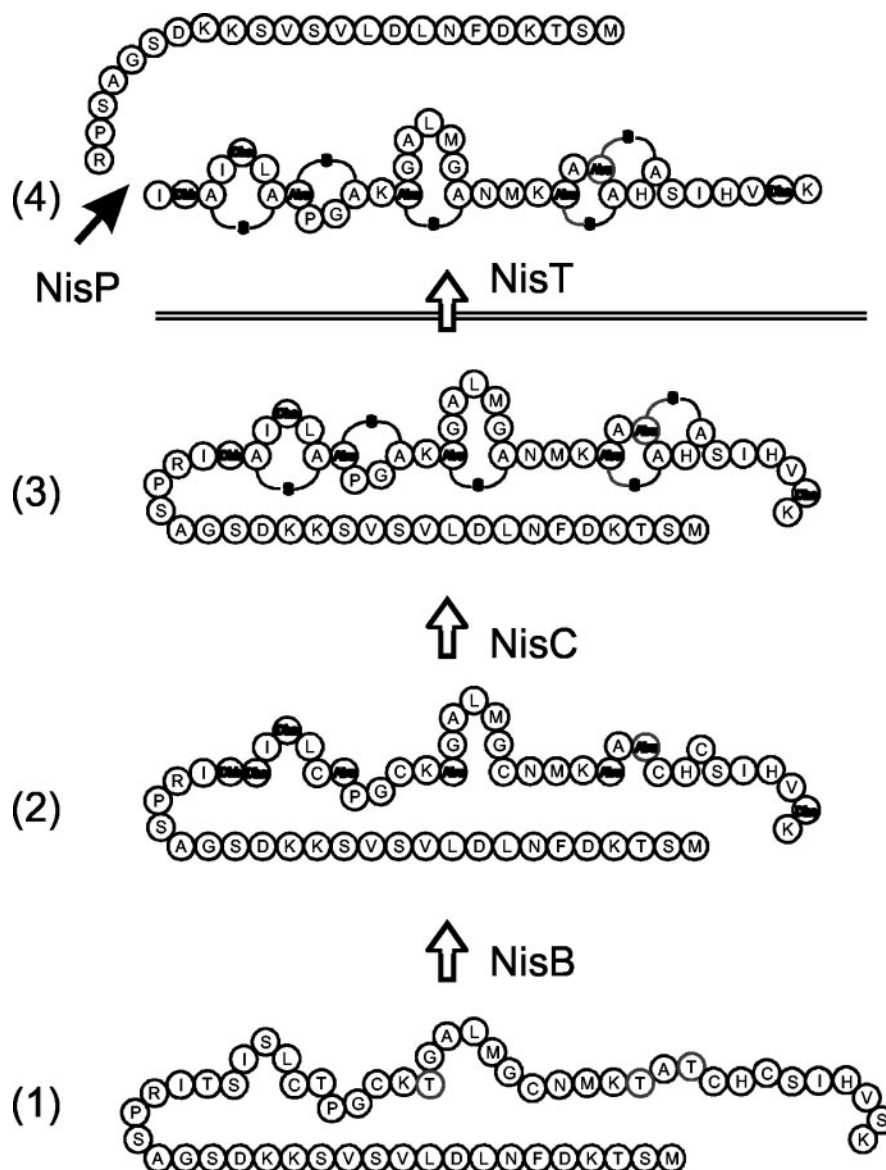


FIG. 1. **Posttranslational modifications of prenisin.** 1, serines and threonines of unmodified prenisin are dehydrated by NisB. 2, the resulting dehydrated prenisin contains dehydroalanines (*Dha*) and dehydrobutyrines (*Dhb*). 3, NisC forms thioether rings between dehydrated residues and cysteines resulting in fully modified prenisin. 4, NisT transports the fully modified prenisin, and NisP cleaves off the leader peptide liberating nisin.

rabbits against the peptide H_2N -STKDFNLDLVSVKKDC-CONH₂ coupled via the cysteine to keyhole limpet hemocyanin. Samples for Western blotting were prepared as follows. Ten ml (20 ml for samples from cells with pLP1vp and pLP1ang) of bacterial culture supernatant was precipitated with 10% trichloroacetic acid, kept on ice for 2 h, pelleted by centrifugation at $18,514 \times g$ during 30 min at 4 °C, washed with 10% trichloroacetic acid and with acetone, and vacuum-dried. Pellets were dissolved in 50 μ l (for TP9703, NZ9700, and PA1001 containing pBMDL5) or 20 μ l of sample buffer and applied on a gel.

Bacterial Strains and Plasmids—Strains and plasmids are listed in Table I. *L. lactis* strains PA1001 and TP9703 were prepared from NZ9000 and NZ9700, respectively, using the pOri gene replacement system (29). In TP9703, the *nisP* start codon of NZ9700 was replaced by a NotI restriction site.

Molecular Cloning—Nisin gene(s) (combinations) were amplified from chromosomal DNA of *L. lactis* NZ9700. DNA amplification was carried out using Expand high fidelity polymerase (Roche Applied Science) or Pfu polymerase (Invitrogen), in the case of insertion or deletion via round-PCR. Plasmid DNA was isolated using the Roche Applied Science kit. DNA was restricted using restriction enzymes from New England BioLabs Inc. Ligation was carried out with T4 DNA ligase (Roche Applied Science). DNA fragments were isolated from agarose gel using the Zymoclean gel DNA recovery kit (Zymo Research, Orange, CA) or from a PCR mix by using the Roche Applied Science PCR purification kit. For intermediate cloning steps, pGEM-T (Promega) was used. Transformation of *Escherichia coli* (*E. coli* DB3.1 (ccdB mutant); *E. coli* DH5 α and *E. coli* TOP10, all obtained from Invitrogen) was carried out using established procedures (30). Electrotransformation of

L. lactis was carried out as described previously (31) using a Bio-Rad Gene Pulser (Bio-Rad). Nucleotide sequence analyses were performed by BaseClear (Leiden, The Netherlands).

Culturing—*L. lactis* was grown in M17 broth (32) supplemented with 0.5% glucose (MG17) with or without chloramphenicol (5 μ g/ml) and/or erythromycin (5 μ g/ml). *E. coli* was cultured in trypton-yeast medium with or without ampicillin (100 μ g/ml) or erythromycin (100 μ g/ml). Preceding mass spectrometry, cells were cultured, and samples were prepared as follows. Overnight cultures of *L. lactis* grown in MG17 broth were diluted 1/100. At an optical density at 660 nm of 0.4, cells were centrifuged, and the medium was replaced by minimal medium (33) with or without 1/1000 volume of filtered (0.4 μ m) overnight *L. lactis* NZ9700 culture medium containing nisin. Incubation was continued for 4 h or overnight, after which mass spectrometry samples were prepared. In the case of cells containing pNGnisBT, 50 ml of medium was subjected to trichloroacetic acid precipitation prior to further analysis. Growth inhibition experiments were performed as described previously (34) but in the absence of Tween.

Mass Spectrometry—Samples were obtained by ZipTip purification (C18 ZipTip, Millipore). ZipTips were wetted and equilibrated with 50% acetonitrile followed by demineralized water. Then peptides were bound and washed with demineralized water, eluted with a solution of 0.1% trifluoroacetic acid with 30 or 50% acetonitrile, vacuum-dried, and stored at -20 °C until analysis. The dried ZipTip eluent was resuspended in 50% acetonitrile containing 0.1% (v/v) trifluoroacetic acid, and 1 μ l was applied to the target. Subsequently, 1 μ l of matrix (10 mg/ml α -cyano-4-hydroxycinnamic acid completely dissolved by mildly heating and vortexing in 50% acetonitrile containing 0.1% (v/v) triflu-

TABLE I
Lactococcus lactis strains and plasmids

Strains and plasmids	Characteristics	Source/reference
NZ9700	<i>nisABTCIPREFG</i> , nisin producer	Ref. 25
NZ9800	Derived from NZ9700 by disruption of <i>nisA</i>	Ref. 21
NZ9743	Derived from NZ9700 by disruption of <i>nisT</i>	Ref. 26
NZ9000	<i>nisRK</i>	Ref. 25
PA1001	Δ acmA (27), Δ htrA (28), derived from NZ9000	This study
TP9703	Δ nisP, derived from NZ9700	This study
pNZ8048 derived plasmids		Ref. 25
pNGnisT	<i>nisT</i> , Em-r ^a or Cm-r ^b	This study
pNGnisTP	<i>nisTP</i> , Em-r or Cm-r	This study
pNGnisP	<i>nisP</i> , Cm-r	This study
pNZnisA-E3	<i>nisA</i> , Em-r	This study
pLP1	The leader of nisin, Cm-r	This study
pLP1ang	An angiotensin-(1–7) variant (NRSYICP) behind the nisin leader, Cm-r	This study
pLP1vp	A vasopressin variant (SYFQNCPRG) behind the nisin leader, Cm-r	This study
pNG-enkT	An enkephalin variant (YTGFC) behind <i>nisA</i> , Em-r	This study
pBMDL5	<i>nisABTC</i> in a gateway plasmid. An inverted repeat is present between <i>nisA</i> and <i>nisB</i> as on the chromosome of NZ9700, Em-r	This study
pBMDL8b	<i>nisABT</i> (S-6P, P-2L <i>nisA</i>) in a gateway plasmid. An inverted repeat is present between <i>nisA</i> and <i>nisB</i> as on the chromosome of NZ9700, Em-r	This study
pNGnisBT	<i>nisBT</i> , Em-r or Cm-r	This study

^a Em-r, erythromycin resistance gene.

^b Cm-r, chloramphenicol resistance gene.

oroacetic acid) was added to the target and allowed to dry. Mass spectra were recorded with a Bruker Biflex III MALDI-time-of-flight mass spectrometer. To maintain high sensitivity, an external calibration was applied. Ethanethiol treatment (35) was applied to confirm posttranslational modification.

Measurement of Leader Peptidase Specificity—NisP-mediated cleavage of peptides was measured by MALDI-TOF¹ MS. Log phase *L. lactis* strains were induced during 4 h or overnight. In the indicated cases, NisP-mediated cleavage was measured after pH-induced ring closure. Ring closure in dehydrated prenisin was achieved by a 1-h incubation at pH 8.0, which was followed by readjustment of the pH to 4.3. Treatment of peptide-containing supernatant with cells of NZ9000 containing pNGnisTP was performed during 4 h followed by MALDI-TOF MS analysis.

Induction Assay—The presence of nisin was also tested using the sensitive GusA assay (6) that monitors the capacity of nisin to induce the nisin promoter.

RESULTS

Detection of the Nisin Leader Peptide—We first investigated whether the nisin leader peptide would remain present in the culture medium of a nisin-producing *L. lactis* NZ9700 strain. Peptides isolated from the supernatant of this strain reacted with anti-leader peptide antibodies (Fig. 2, lane 3). A Coomassie-stained gel of a C18 column elution fraction showed the presence of peptide (Fig. 3A) with a mass of 2350.9 Da (MALDI-TOF MS). An identical peptide (Fig. 3B) was observed when ZipTip-treated supernatant from a nisin-producing *L. lactis* NZ9700 strain, grown overnight in minimal medium (33), was analyzed via mass spectrometry. This mass corresponds to the nisin leader peptide without the initiating methionine. In addition, peaks with masses corresponding to the nisin leader peptide with the initiating methionine (2482.2 Da) and nisin (3354.2 Da) were detected (Fig. 3B). In control incubations with *L. lactis* strain NZ9000, which does not produce nisin, no peaks were observed. A synthetic leader peptide of residues 2–23 gave a mass peak identical to the peak we assigned to the cleaved leader peptide without the initiating methionine. These data for the first time demonstrated the presence of intact nisin leader peptide in the culture medium following secretion and processing.

Thioether Ring Formation by NisBTC—To investigate whether NisBTC are sufficient for thioether ring formation, we cloned the *nisABTC* genes. Both uninduced (Fig. 2, lane 13) and

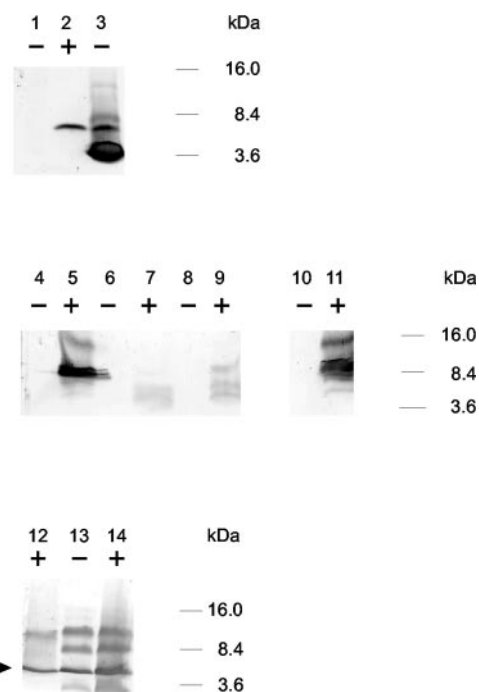
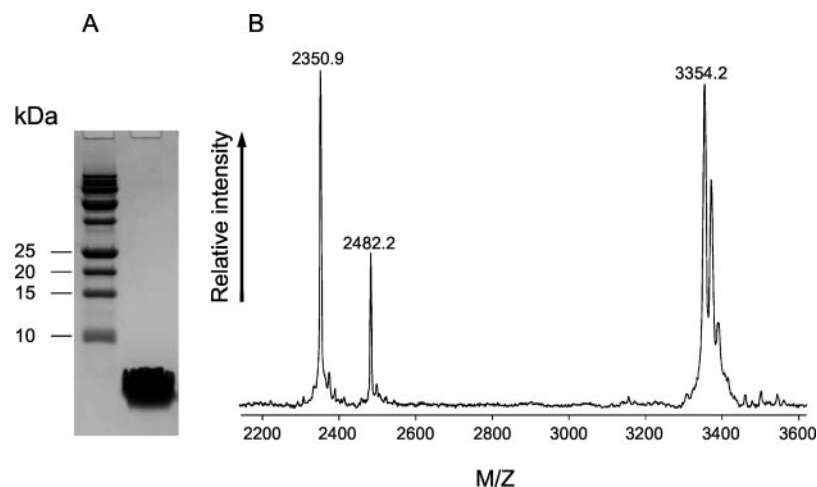


FIG. 2. **Extracellular peptide detection by anti-leader peptide antibodies.** *L. lactis* cells were induced with nisin (+) or not induced (–), incubated for 4 h (NZ9000 containing pNGnisT and pLP1vp) or overnight, and subjected to trichloroacetic acid precipitation and Western blotting. Lanes 1 and 2, strain TP9703; lane 3 strain NZ9700; lanes 4 and 5, strain NZ9000 containing pNGnisT and pNGenK; lanes 6 and 7, strain NZ9000 containing pNGnisT and pLP1vp; lanes 8 and 9, strain NZ9000 containing pNGnisT and pLP1ang; lanes 10 and 11, strain NZ9000 containing pNGnisT and pNZnisA-E3; lane 12, strain NZ9000 containing pBMDL8b; and lanes 13 and 14, strain PA1001 containing pBMDL5. Each experiment was repeated at least three times with similar results.

induced PA1001 (Fig. 2, lane 14) containing pBMDL5 produced prenisin. Apparently, when no inducing nisin was added, some transcription still occurred; this has also been reported previously for wild-type nisin producers (6). Subsequently, we analyzed the prenisin peptides. The uninduced strain PA1001 containing pBMDL5 produced two peptides with masses corresponding to dehydrated prenisin or fully modified prenisin with and without the initiating methionine (5817.4 and 5686.5

¹ The abbreviations used are: MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry.

FIG. 3. Isolation of nisin leader peptide from culture medium. A, leader peptide isolated from *L. lactis* NZ9700 culture medium by binding to Teflon beads and a C18 column was subjected to gel electrophoresis and stained with Coomassie. B, detection of the nisin leader peptide by MALDI-TOF MS. The supernatant of overnight *L. lactis* NZ9700 grown on minimal medium was ZipTip-treated followed by MALDI-TOF MS analysis. Expected average masses ($M+H^+$) are: for the nisin leader peptide residues 2–23, 2352.6 Da; for the nisin leader peptide residues 1–23, 2483.8 Da; for nisin, 3355.2 Da. The experiment shown is a typical result that was repeated more than 10 times with identical results.



Da) and a third peptide corresponding to unmodified prenisin (5833.9 Da). Similar peptide masses were observed after the induction of PA1001 containing pBMDL5 (Table II), but the mass peak of dehydrated or fully modified prenisin (5688.9 Da) was more pronounced.

The trypsin-treated supernatant of induced PA1001 containing pBMDL5 showed a growth-inhibiting activity comparable with that of NZ9700, indicating the presence of fully modified prenisin. Uninduced cells showed a much lower activity. In addition, trypsin-treated culture medium of uninduced and nisin-induced PA1001 cells containing pBMDL5 gained in induction capacity as measured with the GusA assay (6).

The production of fully modified prenisin by PA1001 containing pBMDL5 was confirmed by overlaying agar plate cells with NZ9000 (Fig. 4A) and with NZ9000 containing pNGnisTP (Fig. 4B). As expected, NZ9700 (position 2) produced clear halos with both overlays, whereas PA1001 (position 1) did not form a halo. By contrast, PA1001 containing pBMDL5 (position 3) and TP9703 (position 4) only produced halos when overlaid with NZ9000 containing pNGnisTP (Fig. 4B). Consistent with the lower activity of trypsin-treated TP9703 supernatant, the halos produced by TP9703 were much smaller than those of PA1001 cells containing pBMDL5. These data demonstrate that both induced, and to a lesser extent, uninduced PA1001 cells containing pBMDL5 produce fully modified prenisin, thus showing that the NisBTC enzymes are sufficient for the thioether ring formation.

Export of Dehydrated Prenisin via NisBT—Next, we investigated the functionality of NisBT in the absence of NisC. Strikingly, NZ9000 cells containing pBMDL8B produced prenisin (Fig. 2, lane 12). Mass spectrometry analysis of the supernatant of these cells with plasmid-encoded NisABT (the *nisA* gene of this construct having two leader peptide mutations, S -6 P and P -2 L) demonstrated the production of dehydrated prenisin (5711.2 Da) and unmodified prenisin (5857.0 Da) (Table II). This shows that NisBT can act independently of NisC and also demonstrates that the NisT transport activity is not strictly coupled to full posttranslational modification of prenisin.

Modification and Transport of a Non-lantibiotic Peptide—To investigate whether fusions of the leader with a non-lantibiotic peptide could be modified by NisB and transported by NisT, peptide production by NZ9000 containing pNGnisBT and pLP1ang, encoding a fusion of the leader peptide with NRSY-ICP, was investigated. Both unmodified (3317.9 and 3186.5 Da) and dehydrated fusions of leader peptide with angiotensin-(1–7) (3302.9 and 3169.2 Da), with and without methionine 1, were observed (Table II). To confirm the observed dehydration, prior to analysis, the peptide samples were treated with

ethanethiol, which reacts with dehydro residues. Indeed, after ethanethiol treatment, the peptide with the mass of dehydrated leader angiotensin-(1–7) had disappeared, whereas a peptide corresponding to ethanethiol-modified dehydrated peptide appeared (3235.6 Da). As expected, ethanethiol treatment did not alter the mass of the non-dehydrated peptide (3188.6 Da). These data clearly prove that the fusion of leader peptide with angiotensin-(1–7) was dehydrated and transported by NisBT.

NisT Has a Broad Substrate Specificity—We subsequently determined whether NisT, in the absence of NisBC, is capable of transporting various unmodified fusion peptides containing the nisin leader peptide. Experiments were performed with NZ9000 cells containing two plasmids, one coding for NisT and the second for a leader peptide fusion. Export of unmodified prenisin (Fig. 2, lane 11), unmodified prenisin with a C-terminally fused enkephalin peptide (Fig. 2, lane 5), a fusion of the leader peptide with a vasopressin variant (Fig. 2, lane 7), and a fusion of the leader peptide with an angiotensin variant (Fig. 2, lane 9) was measured using anti-leader peptide antibodies. Mass spectra clearly demonstrated export of unmodified prenisin without the initiating methionine (5833.2 Da), of a fusion peptide of unmodified prenisin with a C-terminal enkephalin variant (6403.2 Da), of a fusion of nisin leader peptide with vasopressin (3405.2 Da), and of a fusion peptide of the nisin leader peptide with angiotensin-(1–7) (3186.6 Da) (Table II). Control experiments without pNGnisT and with strain NZ9743 (26) containing disrupted *nisT* showed no detectable levels of secreted peptide in the culture medium. Furthermore, in the cell fraction of induced NZ9743 cells, an antibody-reactive peptide was detected (data not shown). Taken together, the data clearly demonstrate that NisT can act independently of the other lantibiotic enzymes and further show that the substrate specificity of NisT is much wider than only the fully modified prenisin.

NisP Is Specific for Thioether Ring-containing Prenisin—We measured which leader peptide-containing peptides could be cleaved by the leader peptidase. The leader peptide could neither be cleaved from the leader peptide-angiotensin fusion nor be cleaved from the unmodified or dehydrated prenisin (Table III). However, after keeping the dehydrated prenisin for 1 h at pH 8.0, the leader peptide could be cleaved off. At pH 8.0, thioether rings can be closed spontaneously (36, 37), after which the peptide apparently had become substrate for the leader peptidase. The pH 8.0- and NisP-treated dehydrated prenisin had, however, no antimicrobial activity (data not shown), which indicates that no fully modified prenisin had been formed. Fully modified prenisin itself was also cleaved by the leader peptidase, and a control experiment showed that, in

TABLE II
Modification and export of peptides in *L. lactis*

Cells were induced and grown 4 h (NZ9000 containing pNGnisT and pNGang, NZ9000 containing pNGnisT and pLP1vp) or overnight in minimal medium followed by trichloroacetic acid precipitation and/or direct ZipTip treatment of the supernatant and MALDI-TOF MS. Experiments were repeated at least three times with similar result. Theoretical values are average masses in Da ($M+H^+$).

<i>L. lactis</i> strain	Plasmids	Peptide	Observed mass ($M+H^+$)	Theoretical mass with Methionine 1	Theoretical mass w/o Methionine 1
Da					
NZ9000					
PA1001					
PA1001	pBMDL5 (<i>nisABTC</i>)	Fully modified prenisin	5688.9	5820.0	5688.8
			5820.3		
		Unmodified prenisin	5836.3	5964.0	5832.8
NZ9000	pBMDL8b (<i>nisABT</i>)	Fully dehydrated S-6P, P-2L prenisin	5711.2	5846.1	5714.9
		Unmodified prenisin	5857.0	5990.1	5858.9
NZ9000	pNGnisBT pLP1ang	Leader peptide fused to angiotensin:	3186.5	3317.8	3186.6
			3317.9		
		Dehydrated	3169.2	3299.8	3168.6
			3302.9		
		Dehydrated, ethanethiol addition	3235.6	3362.8	3231.6
		Unmodified, (ethanethiol)	3188.6	3317.8	3186.6
		Unmodified prenisin	5833.2	5964.0	5832.8
NZ9000	pNGnisT pNZnisA-E3				
NZ9000	pNGnisT pNGenkT	Unmodified prenisin C-terminally fused to enkephalin	6403.2	6535.6	6404.4
NZ9000	pNGnisT pLP1vp	Leader peptide fused to vasopressin	3405.2	3537.0	3405.8
NZ9000	pNGnisT pLP1ang	Leader peptide fused to angiotensin	3186.6	3317.8	3186.6

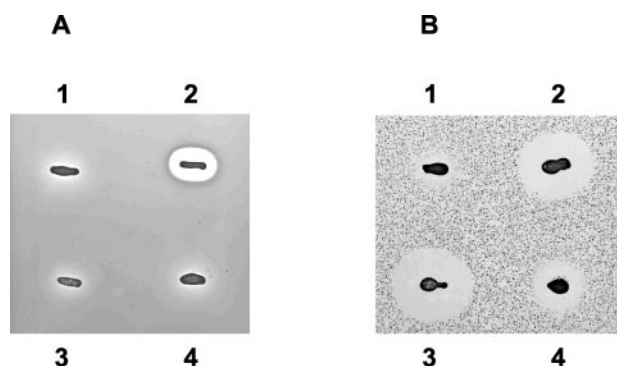


FIG. 4. Leader peptidase activity in NisP producing cells. PA1001 (position 1), NZ9700 (position 2), PA1001 containing pBMDL5 (position 3), and TP9703 (position 4) cells were grown overnight on agar plates that contained no antibiotic next to inducing amounts of nisin. Subsequently, the cells were overlaid with log phase-grown sensitive strain NZ9000 (A) and NZ9000 containing pNGnisTP (B) cells and further grown for one more night. The size of the halo indicates the presence of active nisin processed by NisP. The experiment was repeated three times in duplicate with similar result.

the absence of leader peptidase, the pH 8.0 treatment alone did not liberate the leader peptide (Table III).

To discriminate between NisT-dependent- and -independent NisP activity, a plasmid containing the *nisp* gene was constructed. An antimicrobial activity assay involving fully modified prenisin and mass spectrometry analysis confirmed that the NZ9000 cells containing pNGnisP expressed active NisP as they were able to cleave the leader peptide from externally added fully modified prenisin (data not shown). Hence, NisP can act independently of other lantibiotic enzymes.

DISCUSSION

The leader peptide of nisin may fulfill several functions. First, prior to export, it may have a role in the posttranslational modification and recognition events (17, 38). Second, it is needed for recognition by the transport system, and third, it keeps the lantibiotic in an inactive state until maturation has taken place (4). Here, we demonstrated that the nisin leader

peptide accumulates in the bacterial culture medium of nisin-producing *L. lactis* cells.

The subtilin leader peptide has been shown to act as a translocation signal in *Bacillus subtilis* (39) and in *E. coli* (40). The export of the alkaline phosphatase of *E. coli* when fused with the subtilin leader peptide seemed to be enhanced in the presence of a transporter that is encoded within the subtilin operon (39). When the leader peptidase of subtilin is inhibited by phenylmethylsulfonyl fluoride, accumulation of fully modified pre-subtilin, subtilin, and a series of degradation products in the medium has been observed (41). Mutagenesis studies of the leader peptide of various lantibiotics revealed that some of the leader peptide residues are essential for export and possibly for interaction with the modifying enzymes (42–44). In contrast to the present work, it has also been suggested that fully modified prenisin is the only form of nisin recognized by the transporter (45).

A membrane-associated enzyme complex of NisBTC has been reported to be responsible for dehydration of the serine and threonine residues of prenisin, the thioether ring formation by cross-linking of dehydro residues to cysteines, and the final export step (23). In nisin, Ser-29 is not dehydrated, whereas Ser-33 sometimes escapes dehydration. Overexpression of NisB results in a more frequent dehydration of Ser-33 (22). Here we report that the genes *nisABTC* are sufficient for production and export of fully modified prenisin. Strikingly, we also demonstrate that NisB and NisT suffice to export dehydro residue-containing peptides. This implies that ring formation is not a prerequisite for export. Dehydrated prenisin is, however, not a substrate for NisP, which indicates that further modification is needed before NisP recognizes the prenisin as substrate. Incubation of the dehydrated form of prenisin at pH 8.0 results in spontaneous ring closure (36, 37). Under those conditions, the peptide becomes a substrate of NisP, and the leader peptide can be cleaved off. *L. lactis* NZ9000 with NisABT but without NisC indeed produced the dehydrated prenisin with the dehydroalanines and dehydrobutyrynes present and without the ring closure. The serine and threonine residues in the leader peptide are never dehydrated, as confirmed by mass

TABLE III
The leader peptidase is specific for thioether ring containing prenisin

Cleavage of peptides by NisP was measured by MALDI-TOF MS. NisP was expressed by the peptide-producing cells, or cells expressing NisP were added or, in control experiments, no NisP was present. By incubating for 1 h at pH 8.0, thioether ring closure in dehydrated prenisin was induced. Theoretical values are average masses in Da ($M+H^+$).

<i>L. lactis</i> strain	Plasmid	Post-treatment	Peptide	Observed mass ($M+H^+$)	Theoretical mass after cleavage	Theoretical mass w/o cleavage
Da						
NZ9000	pLP1vp + pNGnisTP		Leader peptide-vasopressin		2352.6	3405.8
PA1001					1072.2	
NZ9000	pLP1ang + pNGnisTP		Leader peptide-angiotensin	3187.1	2352.6	3186.6
NZ9000	pNZnisA-E3 + pNGnisTP		Unmodified prenisin	5836.3	853.0	
					2352.6	5832.8
NZ9000	pBMDL8b (<i>nisABT</i>)	NZ9000/pNGnisTP cells	Dehydrated S-6P, P-2L prenisin	5714.9	3499.2	
					2378.7	5714.9
NZ9000	pBMDL8b (<i>nisABT</i>)	pH 8.0 and NZ9000/pNGnisTP cells	Dehydrated S-6P, P-2L prenisin with 1 or more thioether rings	2377.1	3355.2	
					2378.7	5714.9
PA1001	pBMDL5 (<i>nisABTC</i>)	pH 8.0	Fully modified prenisin	3349.8	3355.2	
PA1001	pBMDL5 (<i>nisABTC</i>)	NZ9000/pNGnisTP cells	Leader peptide and fully modified nisin	5686.3	2352.6	5688.8
					3355.2	
				2351.9	2352.6	5688.8
				3353.9	3355.2	

spectrometry (Fig. 3B). NisBT-expressing cells produced the dehydrated prenisin with 8 dehydrated serine and threonine residues; they also produced a fusion of leader peptide with dehydrated angiotensin-(1–7) (Table II). This implies that bacteria that only contain LanBT (homologs of NisBT) or LanT and the equivalent LanM part (the N-terminal dehydration domain of LanM) can produce peptides with dehydro residues. Therefore, two more amino acids are in principle available as building blocks for the synthesis of novel (poly)peptides with desired properties. Dehydro residues have been reported to be essential for the activity of some bioactive (poly)peptides (46–51). Although the mechanism of NisB action is not known, statistical studies on the variability of the flanking regions of the 8 dehydratable serine and threonine residues in nisin and related lantibiotics suggest that NisB, in contrast to the specific NisP, is equipped with a broad substrate specificity. Engineering of dehydro residues in nisin and Pep5 has been demonstrated (3, 52). Therefore, a wide variety of peptides with dehydro residues might be produced and exported via NisBT. In this context, it is interesting to note that prePep5 fragments with dehydrated residues of Pep5 are exported (53) when the *pepC* gene is largely deleted. However, those studies all concern original lantibiotics, and it would be of interest to introduce such residues in peptides that are normally not modified. Lantibiotic transporters are generally considered to export only specific lantibiotics synthesized by the gene products encoded in the same operon structure. Also, some nisin-subtilin chimeras are exported (54), whereas nisin Z export can be directed by the subtilin leader peptide (55). Those studies, however, all pointed at a rather narrow substrate specificity. Here we demonstrate that the specificity of the NisT transporter is much wider than originally anticipated. Various unrelated peptides, in either a modified form (in the presence of NisB) or an unmodified form (in the absence of NisB), can be secreted provided that they are fused to the leader peptide.

Previous attempts to demonstrate functionally active NisP upon overproduction in *L. lactis* have not been conclusive (4). Here we show that, in the absence of other lantibiotic enzymes, NisP can be functionally expressed in *L. lactis*. The enzyme shows a clear leader peptidase activity on fully modified prenisin. These data furthermore demonstrate that the NisP activity is not coupled to the transport step by NisT. This agrees

with observations that extracellularly added fully modified prenisin is processed by *L. lactis* NZ9800 (4). Remarkably, neither the dehydrated prenisin nor the leader peptide fusions were cleaved by NisP. This strongly suggests that the leader peptidase is specific for thioether ring-containing prenisin. In this respect, the dehydrated prenisin became a substrate for NisP after pH-induced ring closure, which is very suggestive of region and stereospecific closure of one or more rings. Using model peptides, non-enzymatic, stereospecific ring closure has been shown for ring B (36, 37) and ring E (37) of nisin, whereas region specificity and a three-to-one stereo preference was shown for ring A of subtilin (37). These data indicate that production of dehydro residue-containing peptides may be followed by extracellular specific ring closure, e.g. at pH 8.0.

Thioether rings are essential for most lantibiotic activities. The opening of ring A or C (9) or replacement of a thioether ring by a disulfide bridge and reducing it (56) causes a severe loss of activity. In addition, the rings can protect (poly)peptides against proteolytic degradation (3, 4), and their presence may modulate the activity of peptides (2). Active lanthionine analogs of somatostatin and enkephalin have been synthesized chemically (2, 57), but this involved elaborate methods that more easily could be performed by a fermentative route. The transport by NisT of medically relevant therapeutic peptides like enkephalin, vasopressin, and dehydrated angiotensin (variants) compel further research on modifying such peptides by the lantibiotic enzymes.

Summarizing, we have shown that NisBT is sufficient to dehydrate and export the dehydrated non-lantibiotic angiotensin-(1–7) and the dehydrated prenisin and that the NisT transporter is equipped with a wide substrate specificity, transporting various peptides provided they are fused to the nisin leader peptide. Production of peptides via NisBT provides an adequate system to study the substrate specificity of NisB and may enable the synthesis and export of a wide variety of peptides with dehydro residues. This process can then be followed by extracellular stereospecific ring closure to avoid possible export incompatibilities of bulky thioether ring-containing peptides.

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